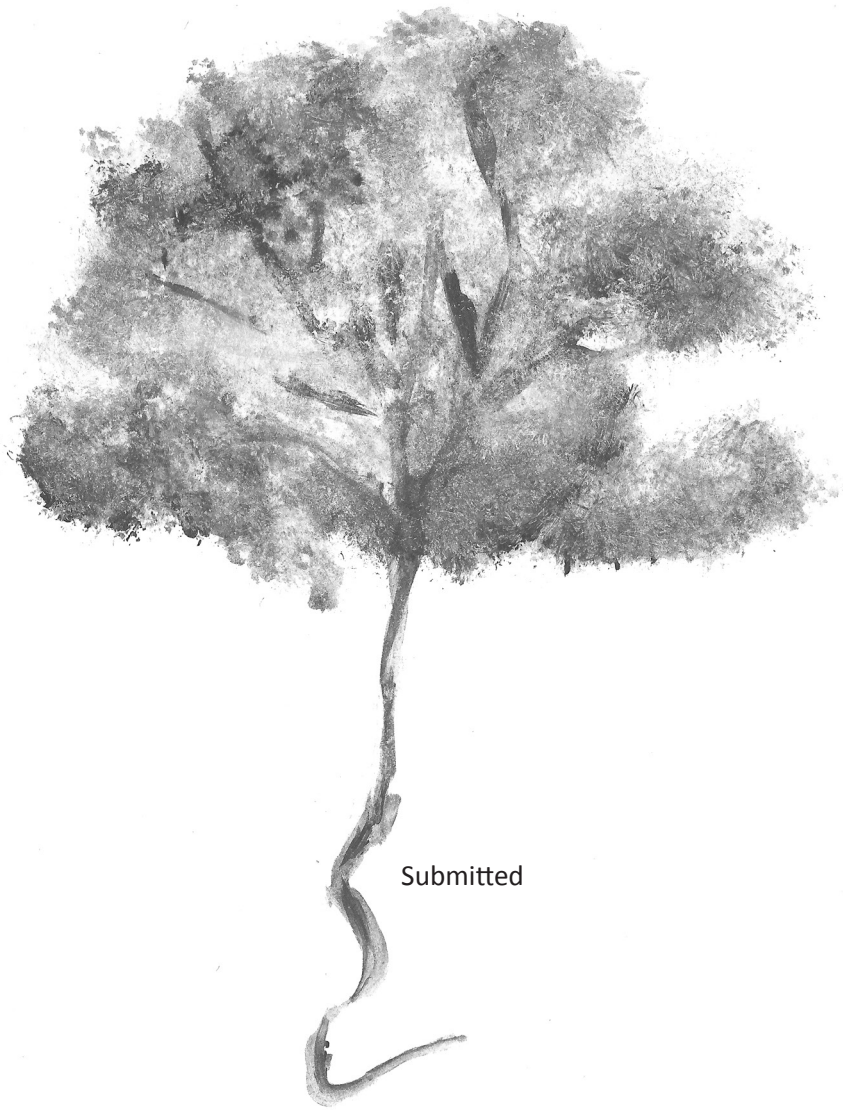
Placental particles in pregnancy and preeclampsia

Göhner, Claudia

Chapter 5

Syncytiotrophoblast extracellular vesicles mediate a tolerance-inducing phenotype in lymphocytes

Claudia Göhner, Justine S. Fitzgerald, Jolien Fledderus, Maja Weber, Ekkehard Schleußner, Udo R. Markert, Marijke M. Faas, Torsten Plösch, Sicco A. Scherjon



Submitted

Abstract

Extracellular vesicles are produced by a plethora of cells during health and disease. In pregnancy, extracellular vesicles might be involved in the immune adaptation of the maternal body towards the semi-allogenic fetus, but they are also under suspicion of being engaged in the pathophysiology of the pregnancy-complication preeclampsia. In this study, we examined the influence of syncytiotrophoblast microvesicles and exosomes on human lymphocytes to shed light on their potential immune involvement in normal and preeclamptic pregnancy. We stimulated whole blood of healthy, nulliparous women with physiologic peripheral plasma concentrations of syncytiotrophoblast microvesicles or exosomes from normal and preeclamptic placentae. The stimulation with syncytiotrophoblast extracellular vesicles from normal placentae induced an activation (increased CD69 expression) of CD8- and CD8+ T cells, regulatory (FoxP3) and memory (CD45RO) T cells as well as an activation (perforin, granzyme B) of natural killer T cells and CD16+CD56++ natural killer cells. Syncytiotrophoblast microvesicles and exosomes induced similar effects, but the effect of exosomes was significantly stronger. This may be due to a partially different molecular load of the two extracellular vesicle subtypes. Syncytiotrophoblast extracellular vesicles from preeclamptic placentae failed to induce the tolerant phenotype in lymphocytes compared to the vesicles from normal placentae. We suggest that the normal placenta uses syncytiotrophoblast microvesicles and exosomes to induce a fetus-tolerant state in the maternal immune system and a protection by memory Treg cells. The preeclamptic vesicles might have lost this protective function, causing a deficit of regulatory cells and therefore the exaggerated inflammatory state in the maternal organism during preeclampsia.

Introduction

Extracellular vesicles (EV) are small membrane-coated particles which are formed by diverse cells, such as endothelial cells, platelets or tumor cells, and are present during health and disease in all body fluids.[1–3] Definitions of EV vary between researchers, but usually they are subdivided into several groups according to their size and way of formation.[4] Often, EV are subdivided into three groups: 1. relatively large macrovesicles or apoptotic bodies, 1 to 5 µm in size; 2. microvesicles (MV) 100 to 1000 nm in size and budding from the plasma membrane; and 3. small nanovesicular exosomes, 30 to 100 nm in size and mostly formed inside intracellular multivesicular bodies which fuse with the plasma membrane to release the exosomes.[5,1] As discussed in several reviews [1–3,5], EV fulfill diverse functions related to angiogenesis, cell survival, coagulation, waste management, cell communication and immune adaptation. It is expected that macrovesicles, MV and exosomes have individual functions, differing from each other.[5,1] Particularly interesting is their immunologic function. It has been shown, that tumor-derived MV may contribute to the establishment of an immune-privileged side to protect the tumor from the hosts immune system by upregulation of regulatory T (Treg) cells.[6] Furthermore, exosomes might provide an immune regulatory advantage for recipients of organ transplants by mediating allograft tolerance.[7] Also during pregnancy, EV are believed to be involved in the immune adaptation of the maternal body to the semi-allogenic fetus and placenta.[3]

During pregnancy, diverse immune changes can be observed in the maternal body which ensure the successful establishment and maintenance of pregnancy. These adaptations of the maternal immune system lead to changes in the immune balance during pregnancy, with an increased systemic inflammatory state of the maternal body, while at the local, placental level immune suppression is induced.[8–10] In peripheral blood, it has been shown that the number and activation of monocytes and granulocytes is increased in pregnancy [8,11] and that the phenotype of monocytes is shifted from CD14++CD16- classical monocytes to CD14++CD16+ intermediate and CD14+CD16++ non-classical monocytes [12]. As a sign of immunoregulation, Treg have been found to be increased during pregnancy, both in peripheral blood and locally at the placenta, mediating tolerance towards the fetus in human [13] and mice [14]. T helper (Th) cells show a shift of the Th1/Th2 ratio towards Th2 cells.[15] In contrast, natural killer (NK) cells are reduced in pregnancy, but together with natural killer T (NKT) cells also show a shift to the production of Type2 cytokines.[16–18] The syncytiotrophoblast (STB), as the outer fetal layer of the placenta facing the maternal blood, secretes STB macrovesicles, MV and exosomes which enter the maternal blood to reach their target cells, e.g. maternal immune cells.[3,19] The number of STB EV increases during the course of pregnancy [20], suggesting that these STB EV may involve in immune adaptation to pregnancy.

STB EV are strongly associated with the pathophysiology of preeclampsia (PE), a severe pregnancy-complication occurring in 3-5% of the pregnancies.[21,22] PE is characterized by its major symptoms hypertension and proteinuria; but also by a shallow placentation associated with reduced blood flow to the placenta and oxidative stress as well as local hypoxia in the placenta; endothelial dysfunction and an exaggerated inflammatory state in the maternal body.[23] For example, monocytes are further activated compared with normal pregnancy. The numbers of Treg cells are reduced peripherally [24] and locally in the decidua [25] and the immunity is affected by a type 1 shift in Th, NK and NKT cells as compared to normal pregnancy.[12,16,26] It has been shown that the amount of STB EV is increased during PE compared to normal pregnancy [27] and that the molecular load of STB EV from normal and preeclamptic placentae differs from each other.[28]

In this study, we analyzed the impact of STB EV on peripheral lymphocyte subpopulations to shed light on whether STB EV might be involved in the immune adaptations as apparent both in normal and preeclamptic pregnancy. We compared the function of STB MV and exosomes gathered separately from the same placentae to each other to assess a potentially different functional profile of both STB EV groups. Since STB EV seem to have a functional involvement in the pathophysiology of PE, we also compared STB EV from normal and preeclamptic placentae to each other to clarify a potential change of function from normal to diseased STB EV. Therefore, whole blood of non-pregnant female volunteers was incubated with or without STB MV or exosomes from normal placentae or from preeclamptic placentae. Using flow cytometry, we measured the percentual distribution of CD8- and CD8+ T cells (CD3+), Treg cells (FoxP3+), Th cells (Th1 - T-bet+, Th2 - CD294+ and Th17 - RoRγt+), NKT cells (CD3+CD56+) and NK cells (CD16++CD56+, CD16+CD56++). Additionally, we measured the activation (CD69) and memory function (CD45RO) of T cells, and, as it has been described that NK and NKT cells express perforin and granzyme B to mediate their functions [29,30], the expression of perforin and granzyme B on NK and NKT cells.

Materials and Methods

Ex vivo placenta perfusion

Experiments with placental tissue were approved by the ethics committee of the University Hospital Jena, Germany and informed consent was obtained from women involved. Four normal and two preeclamptic placentae were collected after vaginal delivery or elective caesarian section and *ex vivo* placenta perfusion began within 20 min after delivery. Perfusion suspension was composed as follows: per 1 l NCTC-135 (with L-glutamine, without phenol red, without vitamin B12, without sodium hydrogen carbonate, AppliChem GmbH) 0.5 l Earl's buffer, 60 g BSA (MP Biomedicals LLC), 15 g dextran (FP 40, SERVA electrophoresis GmbH), 2 g D-glucose (water-free, Merck KGaA), 0.38 g Amoxicillin (Sigma-Aldrich Chemie GmbH) and 0.75 ml heparin (Heparin-Natrium-25.000, 25,000 i.E./5 ml, Ratiopharm GmbH) were added. Earl's buffer contained 111.225 mM NaCl (Carl Roth GmbH + Co.KG), 5.365 mM KCl (Merck KGaA), 1.015 mM NaH2PO4 (Merck KGaA), 26.187 mM NaHCO3 (Merck KGaA), 811.425 µM MgSO4*7 H2O (Merck KGaA), 1.36 mM CaCl2*2 H2O (Merck KGaA). The pH of the perfusion suspension was adjusted to pH = 7.4 with NaOH (Carl Roth GmbH + Co.KG), the suspension was filtered using a 0.8/0.2 µm filter (AcroPak™ 200 Capsules with Supor® Membrane, Pall Corporation) and frozen at -20 °C until *ex vivo* perfusion of an isolated cotyledon. A proper cotyledon was chosen macroscopically and cannulas were introduced into the fetal main vein and artery of the cotyledon to perfuse as during fetal circulation. Perfusion started with 0.3 mL/min perfusion speed, using perfusion solution purged with 95 % nitrogen/5 % carbon dioxide. The cotyledon was put into a perfusion chamber and cannulas were introduced into the intervillous space by cautious penetration of the decidua to perfuse the maternal circulation. The maternal circuit was perfused at 12 mL/min with perfusion suspension purged with technical air. After connecting the maternal circulation, the fetal flow was slowly increased to its final speed at 3 mL/min to avoid pressure peaks. After a 30 min rinsing period, perfusion was performed for 120 min, the perfusion suspension was collected and centrifuged for 10 min at 380 g at room temperature (RT) to pellet cells. The supernatant was frozen at -80 °C until separation of STB MV and exosomes.

Quantification of STB EV

In this study, we aimed to perform stimulation experiments of blood samples with STB MV and exosomes at a plasma STB EV concentration in late pregnancy. We quantified the peripheral STB EV plasma concentration (consisting of STB MV and exosomes) in women at 36 (+/-1) weeks of gestation (manuscripts in preparation, see chapter 3 of this thesis) recently, using the enzyme-linked sorbent assay as previously described.[31] STB MV and exosomes were isolated from suspension of

ex vivo placenta perfusion, and we assumed that the ratio of STB MV to exosomes is similar in plasma from late pregnant women and the perfusion suspension. STB EV (consisting of STB MV and exosomes) were quantified in the supernatant of the perfusion suspension using the enzyme-linked sorbent assay as previously described. [31]. For stimulation of whole blood with perfusion-derived STB MV or exosomes, the concentration of STB EV was adjusted to the plasma STB EV concentration in late pregnancy.

Separation of syncytiotrophoblast microvesicles and exosomes from perfused perfusion suspension

To isolate STB MV and exosomes from the perfusion suspension, the suspension was slowly thawed at 4 °C. Cell debris was pelleted in 38 ml of the perfusion suspension by centrifugation for 10 min at 10.000 g at 4 °C. All following centrifugations were performed in ultra-clear centrifuge tubes (Beckman Coulter, Krefeld, Germany). STB MV were pelleted for 30 min at 18.900 g at 4 °C and the supernatant was filtered with a 0.8/0.2 µm double filter (Acrodisc PF 32 mm Syringe Filter with 0.8/0.2 µm Supor Membrane, Pall Corporation, Port Washington, NY, USA). Exosomes were pelleted from the filtrate by ultra-centrifugation for 70 min at 100.000 g at 4 °C to pellet exosomes. The STB MV pellet and exosome pellet were each washed in 1 % BSA (Fraction V, MP Biomedicals LLC, Solon, OH, USA) in 0,05 % Tween20-PBS (Polyoxyethylene(20)-sorbitan-monolaurate, Dulbecco's phosphate buffered saline; both Sigma-Aldrich Chemie GmbH, Hamburg, Germany) and pelleted again for 30 min at 18.900 g at 4 °C or for 70 min at 100.000 g at 4 °C, respectively. Pellets were re-suspended 200 µl of 1 % BSA in 0,05 % Tween20-PBS (pH = 7,4 Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA), aliquoted and stored at -80 °C until usage.

Blood stimulation and flow cytometric analysis

Stimulation experiments with human whole blood (Lithium-Heparin, BD Vacutainer, LH 170 I.U., BD, Plymouth, United Kingdom) were approved by the medical ethical committee of the University Medical Center Groningen, the Netherlands and informed consent was obtained from respective women. Peripheral venous blood samples were collected between days 5 to 7 from the start of the current cycle from healthy nulligravida. Inclusion criteria were: 18 to 40 years of age, body-mass-index between 18 and 30. Exclusion criteria were: smoking, use of an intra-uterine device, diagnosed infertility, immune-related disorders, flu-like symptoms within four weeks before blood donation, medication other than folic acid or oral contraceptives.

STB MV and exosomes from each placenta were used to stimulate blood from 3 different donors for 24 h at 37 °C, 5 % CO_2. Blood was aliquoted into 12 well-

plates at 3 ml per well and stimulated with STB MV or exosomes adjusted to the peripheral plasma concentration in late pregnancy (see Quantification of STB EV). As STB MV and exosomes were re-suspended in 1 % BSA (Sigma-Aldrich Chemie GmbH) in 0,05 % Tween20-PBS, equivalent volumes of 1 % BSA in 0,05 % Tween20-PBS were applied as control stimulation.

After the stimulation, whole blood was diluted in an equivalent volume of RPMI 1640 medium (Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA) and red blood cells were lysed twice in 10 mL ammonium chloride buffer (155.17 mM NH4Cl, 9.99 mM KHCO3, 0.56 mM C10H18N2Na2O10, Clinical Pharmacy, University Medical Center Groningen, Groningen, The Netherlands) per 1 mL whole blood for 10 min at 4 °C. All centrifugation steps were performed for 4 min at 4 °C at 560 g and all incubations were performed in the dark. Cells were re-suspended in wash buffer (PBS with 2 % Fetal calf serum, Greiner Bio One International GmbH, Alphen a/d Rijn, The Netherlands), filtered (Falcon® 5mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap, 35 µm nylon mesh, Corning, Corning, NY, USA) and counted with a TC20™ Automated Cell Counter (BioRad, Hercules, CA, USA). Per staining, 1,000,000 cells were blocked in wash buffer with 20 % rat serum (Normal Rat Serum, Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) for 20 min at 4 °C. Next, cells were stained for 30 min at 4 °C in 50 µL of the extracellular antibody mix (table 1) in wash buffer with 5 % rat serum. Afterwards, cells were washed three times with wash buffer, fixed with 200 µL BD FACS Lysing Solution (BD Biosciences, Franklin Lakes, NJ, USA) for 30 min at room temperature and permeabilized by three times washing in 200 µL BD Perm/Wash (BD Biosciences). Next, cells were stained for 30 min at 4 °C in 50 µL of the intracellular antibody mix (table 1) in Perm/Wash with 5 % rat serum. Isotype controls were used at the same antibody concentration. Unstained controls were incubated in wash buffer and Perm/Wash with 5 % rat serum only. Flow cytometric analysis was performed on a BD FACSVERSE flow cytometer (BD Biosciences) with gating as shown in Figure 1.

Statistics

STB EV from each placenta were used to stimulate blood from 3 different donors. Donors differed within the group of healthy placentae (nplacenta = 4, ndonor = 12) and within the group of PE placentae (nplacenta = 2, ndonor = 6). To compare the effects of STB MV and exosomes to control and to each other, the non-parametric Friedman test for related samples was done first. Groups (% of gated cells) which reached $p<0.05$ in the Friedman test were reanalyzed with the non-parametric Wilcoxon signed rank test for two related samples to compare the treatment effects: control versus STB MV, or control versus exosomes, or STB MV versus exosomes. P-values below 0.05 were considered significant. Outliers were identified based on their standard score following the rules of the Grubbs test for critical values.

Table I: Antibody panels for flow cytometric analysis

	Panel 1			Panel 2			Panel 3		
	Antigen	Clone/ isotype	V [µL]	Antigen	Clone/ isotype	V [µL]	Anti-gen	Clone/ isotype	V [µL]
Extracellular	CD3 Pacific Blue#	UCHT1/ mouse IgG1κ	2	CD3 Pacific Blue#	UCHT1/ mouse IgG1κ	2	CD3 Pacific Blue#	UCHT1/ mouse IgG1κ	2
	CD8 FITC*	SK1/ mouse IgG1κ	2	CD8 FITC*	SK1/ mouse IgG1κ	2	CD8 FITC*	SK1/ mouse IgG1κ	2
	CD45Ro PE*	UCHL1/ mouse IgG2ακ	4	CD16 PeCy7*	eBioCB16/ mouse IgG1κ	4	CD294 APC-Cy7#	BM16/ rat IgG2ακ	1.2
	CD69 PeCy7*	FN90/ mouse IgG1κ	1	CD56 APC eFlour780*	CMSSB/ mouse IgG1κ	3			
Intracellular	Perforin PerCP-Cy5.5#	dG9/ mouse IgG2κ	5	Perforin PerCP-Cy5.5#	dG9/ mouse IgG2κ	5	T-bet PeCy7*	eBio4B10/ mouse IgG1κ	0.3
	FoxP3 APC*	PCH101/ rat IgG2ακ	2	Granzyme B PE*	GB11/ mouse IgG1κ	2.5	RoRγt PE*	B2D/ rat IgG1κ	1

The table shows the antigens which were targeted in every panel, the clone in which the respective antibody was produced, the isotype of the respective antibody and the antibody volume (V) used to stain 1,000,000 cells in 50 µL. * eBioscience, Affymetrix, San Diego, CA, USA; # ITK Diagnostics BV, Uithoorn, The Netherlands

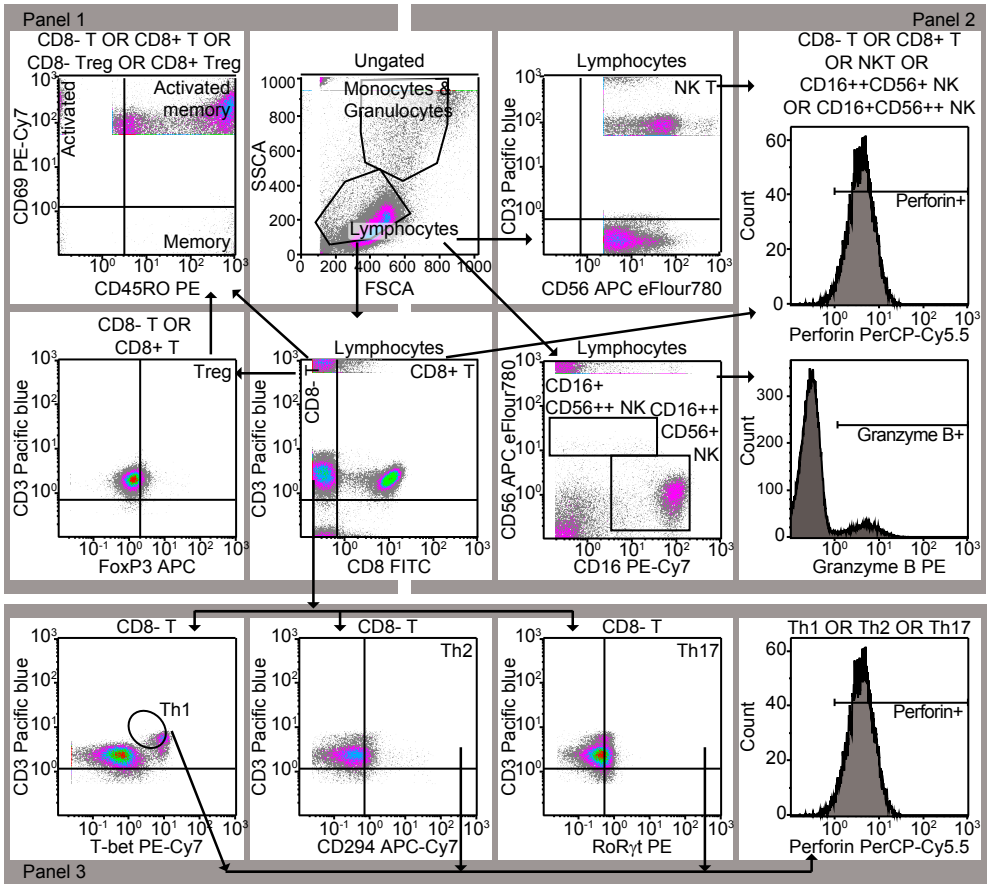


Figure 1: Gating strategy for the flow cytometric analysis of T cells, NKT cells and NK cells after stimulation with syncytiotrophoblast extracellular vesicles

Based on the size (FSCA) and granularity (SSCA), lymphocytes have been distinguished from monocytes & granulocytes. Gated lymphocytes have been further subdivided to CD8- T cells (CD3+CD8-), CD8+ T cells (CD3+CD8+), NKT cells (CD3+CD56+) and NK cells (CD16++CD56+ and CD16+CD56++). In panel 1, CD8- and CD8+ T cells have been analyzed for FoxP3 expression as a marker for Treg cells. Following, CD8- T cells and CD8+ T cells as well as CD8- Treg and CD8+ Treg were analyzed for activation (CD69+) and memory function (CD45RO+). In panel 2, CD8- T cells, CD8+ T cells, NKT cells, CD16++CD56+ NK cells and CD16+CD56++ NK cells have been analyzed regarding their expression of Perforin and Granzyme B as marker for activity/cytotoxicity of these cells. In panel 3, CD8- T cells have been further subdivided down for analysis of their Th cell composition into Th1 (T-bet), Th2 (CD294) and Th17 (RoR□t) cells and also in these subsets have been analyzed for Perforin expression.

Results

STB MV and exosomes from normal placentae activate T cell populations.

In control stimulation of whole blood with BSA-Tween20-PBS, we observed 48 % of CD8- T cells of which 7 % were Treg cells (FoxP3+), and 24 % of CD8+ T cells of which 1.4 % were Treg cells (FoxP3+) in the lymphocyte gate (Figure 2). Additionally, we observed 5.2 % Th1, 1.7 % Th2 and 0.7 % Th17 cells in the CD8- T cell gate (data not shown). Stimulation of whole blood with either STB MV or exosomes from normal placentae neither significantly altered the percentage of CD8- T cells or CD8- Treg cells nor the percentage of CD8+ T cells or CD8+ Treg cells (Figure 2) or Th cells (data not shown). However, staining for CD69 revealed that stimulation with STB MV or exosomes from normal placentae increased the percentage of activated CD8- T cells, activated CD8- memory T cells, and activated CD8+ memory T cells (Figure 2). Moreover, normal exosomes increased the percentage of activated CD8- memory Treg cells and activated CD8+ memory Treg cells, and decreased the percentage of CD8+ T cells (Figure 2). Additionally, stimulation with either normal STB MV or exosomes induced an increased perforin expression in overall CD8- and CD8+ T cell, but not in CD8- and CD8+ Treg cells (data not shown). In the activation of CD8- and CD8+ T cells and Treg cells, the effect of exosomes from normal placentae was significantly stronger than the effect of STB MV from normal placentae.

STB MV and exosomes from normal placentae activate NKT cells and CD16+CD56++ NK cells by induction of the expression of perforin and granzyme B.

In control stimulation of whole blood with BSA-Tween20-PBS, we observed about 5 % NKT cells, 13 % CD16++CD56+ NK cells and 0.6 % CD16+CD56++ NK cells (Figure 3) in the lymphocyte gate. All NKT cell and NK cell populations were found to express perforin and granzyme B in control stimulations (NKT cells: 36 % perforin+, 36 % granzyme B+; CD16++CD56+ NK cells: 91 % perforin+, 90 % granzyme B+; CD16+CD56++ NK cells: 54 % perforin+, 35 % granzyme B+) (Figure 3). The stimulation with either STB MV or exosomes from normal placentae significantly increased the percentage of NKT cells and the percentage of perforin+ NKT cells (Figure 3). Additionally, the stimulation with exosomes from normal placentae significantly increased the percentage of perforin+ CD16+CD56++ NK cells and of granzyme B+ CD16+CD56++ NK cells (Figure 3). Regarding the increase of the percentage of perforin+ and granzyme B+ cells, the effect of exosomes from normal placentae was significantly stronger than the effect of STB MV from normal placentae.

STB MV and exosomes from preeclamptic placentae activate CD8+ memory T cells.

In comparison to control stimulation of whole blood with BSA-Tween20-PBS, stimulation of whole blood with either STB MV or exosomes from preeclamptic placentae did not significantly affect the percentage of CD8- and CD8+ T cells, CD8- and CD8+ Treg cells (Figure 4) or Th1, Th2 or Th17 cells (data not shown). It also neither significantly activated CD8- T cells, CD8- Treg or CD8+ Treg (Figure 4) nor induced perforin expression in CD8- and CD8+ T cells or CD8- and CD8+ Treg cells (data not shown). However, stimulation with either STB MV or exosomes from preeclamptic placentae induced a significant increase of activated CD8+ memory T cells (Figure 4).

STB MV and exosomes from preeclamptic placentae induce perforin expression in NKT cells and reduce CD16++CD56+ NK cells.

In comparison to control stimulation of whole blood with BSA-Tween20-PBS, stimulation of whole blood with either STB MV or exosomes from preeclamptic placentae did not significantly affect the expression of perforin and granzyme B in CD16++CD56+ NK cells or CD16+CD56++ NK cells (Figure 5). However, stimulation with either STB MV or exosomes from preeclamptic placentae significantly increased the percentage of perforin+ NKT cells (Figure 5). Additionally, stimulation with exosomes from preeclamptic placentae significantly reduced the percentage of CD16++CD56+ NK cells (Figure 5).

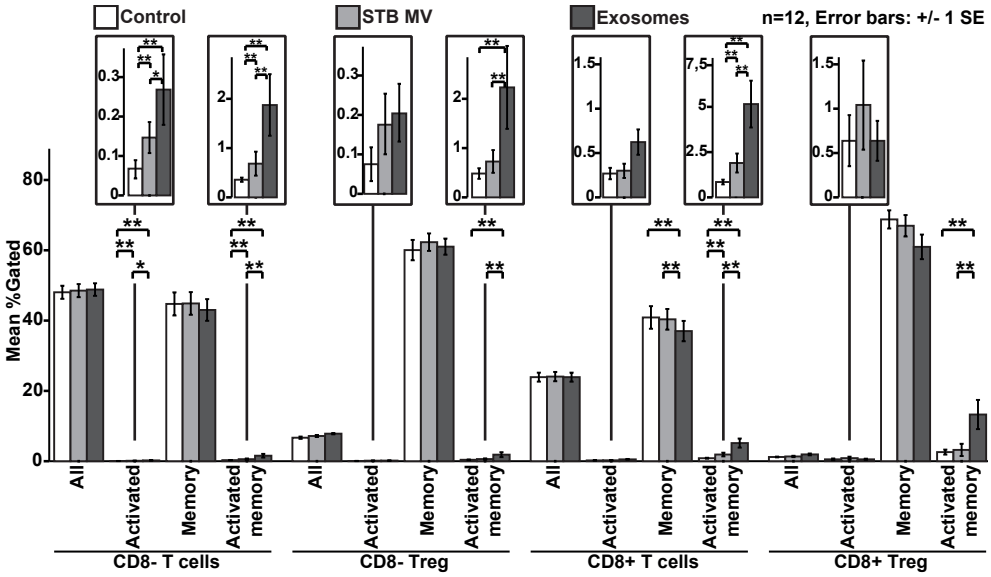


Figure 2: Percentage of activated and memory CD8- and CD8+ T cells after stimulation with normal syncytiotrophoblast extracellular vesicles

Whole blood was stimulated for 24 h with normal syncytiotrophoblast microvesicles (STB MV) or exosomes and the percentage of CD8- and CD8+ T cells as well as CD8- and CD8+ Treg cells was analyzed flow cytometric (whole gated population labeled with "all"). Further, it has been analyzed which percentage of these cells are activated (CD69+), show a memory function (CD45Ro+) or are activated memory cells (CD69+ and CD45RO+). Wilcoxon Signed Rank test: * p<0,05, ** p<0,01

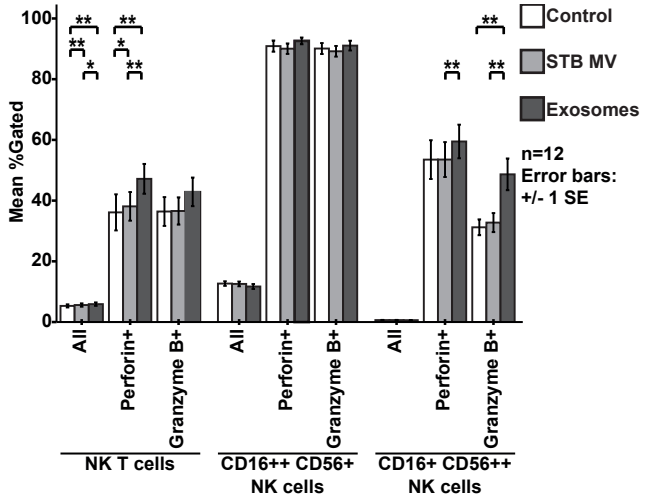


Figure 3: Expression of Perforin and Granzyme B by NKT cells and NK cells after stimulation with normal syncytiotrophoblast extracellular vesicles

Whole blood was stimulated for 24 h with normal syncytiotrophoblast microvesicles (STB MV) or exosomes and analyzed flow cytometric. The percentage of the whole populations of NKT cells, CD16++CD56+ NK cells and CD16+CD56++ NK cells is labeled with "all", respectively. The percentage of Perforin+ and Granzyme B+ cells has been assessed within the whole populations. Wilcoxon Signed Rank test: * p<0,05, ** p<0,01

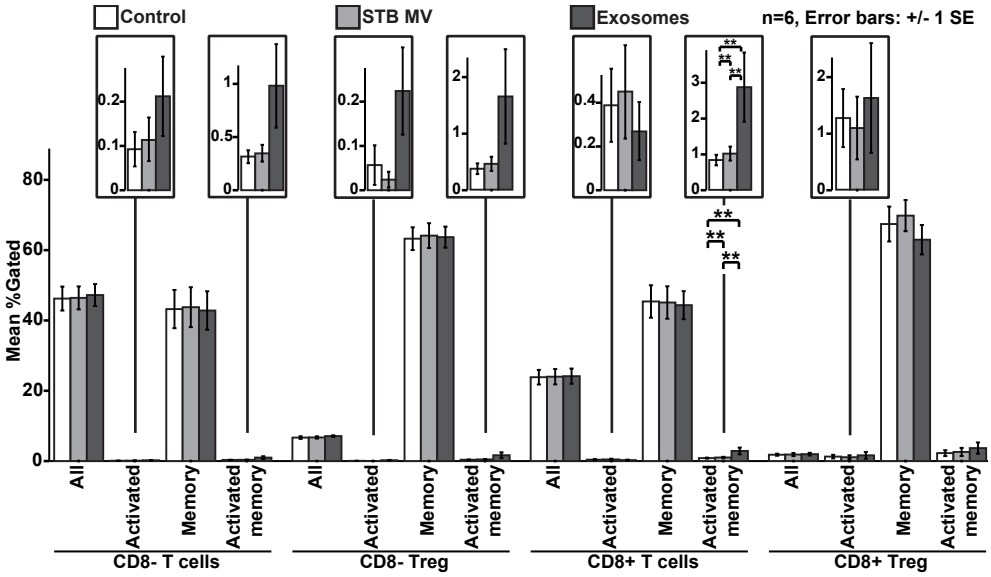


Figure 4: Percentage of activated and memory CD8- and CD8+ T cells after stimulation with preeclamptic syncytiotrophoblast extracellular vesicles

Whole blood was stimulated for 24 h with preeclamptic syncytiotrophoblast microvesicles (STB MV) or exosomes and the percentage of CD8- and CD8+ T cells as well as CD8- and CD8+ Treg was analyzed flow cytometric (whole gated population labeled with "all"). Further, it has been analyzed which percentage of these cells are activated (CD69+), show a memory function (CD45Ro+) or are activated memory cells (CD69+ and CD45RO+). Wilcoxon Signed Rank test: * p<0,05, ** p<0,01

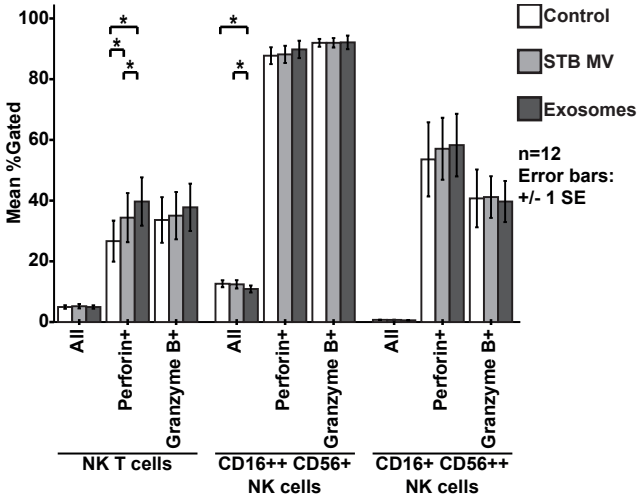


Figure 5: Expression of Perforin and Granzyme B by T cells, NKT cells and NK cells after stimulation with preeclamptic syncytiotrophoblast extracellular vesicles

Whole blood was stimulated for 24 h with preeclamptic syncytiotrophoblast microvesicles (STB MV) or exosomes and analyzed flow cytometric. The percentage of the populations of NKT cells, CD16++CD56+ NK cells and CD16+CD56++ NK cells is labeled with "all", respectively. The percentage of Perforin+ and Granzyme B+ cells has been assessed within the whole populations. Wilcoxon Signed Rank test: * p<0,05, ** p<0,01

Discussion

In this study, we aimed to assess the effect of STB MV and exosomes from normal and preeclamptic placentae on the distribution and cytotoxicity of peripheral lymphocyte populations. We showed that both CD8- and CD8+ T cells, and the memory T cell fraction, became activated by stimulation with normal STB EV as apparent by an increased percentage of CD69+ cells. Furthermore, the percentages of perforin+ NKT cells and CD16+CD56++ NK cells and the percentages of granzyme B+ CD16+CD56++ NK cells were increased after stimulation with normal STB EV which indicates an increased activity of also these cells. The percentages of Th1, Th2 and Th17 cells were not significantly altered by stimulation with normal STB EV.
In accordance with our hypothesis, the stimulation of whole blood with normal STB MV or exosomes induced an activation (CD69) and increased cytotoxicity (perforin) of CD8- and CD8+ T cells. The activation of T cells was especially apparent in memory cells (CD69+CD45RO+) and memory Treg cells (FoxP3+CD69+CD45RO+). From the present study, it is unknown why especially memory T cell became activated, but it could be related to the fact that predominantly CD8+ memory cells are recruited to the decidua to protect the fetus from harmful infection.[32,33] Additionally, Treg cells have been described to be essential for the successful maintenance of pregnancy and for the tolerance induction towards the semi-allogenic fetus while showing an activated memory-like phenotype.[9,34] Induction of memory Treg cells by STB EV may be a mechanism to sustain protective regulatory memory to fetal antigens, which may be important for future pregnancy.[35]

This tolerance-inducing phenotype of Treg cells by STB EV might be supported by NKT and NK cells, which increasingly produce perforin and granzyme B in response to the stimulation with STB EV from normal placentae. NKT and NK cells use, amongst others, perforin and granzymes to kill their target cells, e.g. tumor cells.[29,36,37] Within the NK cell subpopulations, the peripheral CD16+CD56++ NK cells are described to be rather regulatory and tolerance inducing [30], and in our experiments especially this NK cell population was influenced by normal STB EV. We suggest that the increased activity (in terms of perforin and granzyme B production) of regulatory NKT and NK cells might support Treg cells in regulating the immune response during pregnancy and thus preventing an immune reaction against the semi-allogenic fetus by suppressing effector cells.

Based on the differing modes of formation and molecular load of MV and exosomes, it is generally expected that these EV types also have different functions, with MV being rather activating, and exosomes being rather tolerance-inducing.[3] Comparison of the effects of STB MV and exosomes from normal placentae matched from the same placentae revealed that both STB EV subtypes seem to work similarly in modulating the maternal immune response. However, the effect of exosomes was significantly more pronounced than the effect of STB MV. The stronger effect of the exosomes might be based on a specific molecular load different from that of

STB MV. Unfortunately, to our knowledge, no studies have been published yet which compare the molecular load of STB MV and exosomes. We performed nanoparticle tracking analysis on our samples of STB MV and exosomes from *ex vivo* placenta perfusion suspension (unpublished data), and the results indicate that the ratio of STB V to exosomes is about 1 in perfusion suspension. Thus, it is unlikely that the number of exosomes was higher than the number of STB EV in our study and a difference in numbers of STB MV and exosomes cannot be the cause of the stronger exosomal effect.

We assumed that the functionality of STB EV might be altered from healthy to preeclamptic pregnancies. Compared to the results of stimulation with STB EV from normal placentae, STB EV from preeclamptic placentae failed to activate (memory) CD8- and CD8+ T cells and Treg cells or to induce perforin+ and granzyme B+ CD8- T cells, CD8+ T cells and CD16+CD56++ NK cells. Also, STB EV from preeclamptic placentae did not induce a shift of Th cells towards Th1 immunity. The pregnancy-complication PE is, amongst others, characterized by an exaggerated inflammatory state, a shift from type 2 to type 1 immunity and reduced numbers of peripheral and local Treg cells.[15,16,23,24] STB EV are believed to be involved in the pathophysiology of PE. It has been shown that the number of STB EV is increased in PE compared to normal pregnancy and that also the molecular load of STB EV from preeclamptic placentae is altered compared to STB EV from normal placentae.[27,28] In our study, STB EV from preeclamptic placentae seem to have lost the regulative function of STB EV from normal placentae to induce activation regulatory cells such as of Treg, NKT and CD16+CD56++ NK cells. This might cause a deficit of regulatory cells and therefore may allow an increased inflammatory state in the preeclamptic pregnancy. Therefore, STB EV may not actively induce the exaggerated inflammatory response in PE (see chapter 4), but rather favor the development of these symptoms by not inducing regulatory Treg, NKT and NK cells.

As reviewed several times, the risk of developing PE is increased under several circumstances.[23,38] Especially striking is the fact that the risk of developing PE is increased in nulliparous women and even more in multiparous women who developed PE in a previous pregnancy.[39] Our data suggest that STB EV from normal placentae may induce a tolerance-mediating memory-like phenotype in Treg cells of nulliparous women. STB EV from preeclamptic placentae failed to induce this phenotype in Treg cells of nulliparous women. We speculate that STB EV from preeclamptic placentae might not be able to establish a memory function in Treg cells, which under physiological conditions may provide a beneficial protection to the maternal organism with respect to a further pregnancy. Since this memory protection is not established, women might be predisposed to experience PE again in a following pregnancy.

In conclusion, we showed that STB EV from normal placentae may be involved in the induction of immunologic tolerance of the semi-allogenic fetus necessary for maintaining pregnancy by especially influencing memory T cells and

memory Treg cells. In contrast to the initial expectations, STB MV and exosomes did not show opposite functions but rather mediated similar effects. However, STB EV from preeclamptic placentae seem to lose the regulatory and protective function of STB EV from normal placentae, which in the end might provoke the exaggerated inflammatory response during PE. To better understand the described processes, a deeper understanding of the molecular mechanisms of the interaction of STB EV with their target cells and a comprehensive comparison of the molecular load and functionality of STB EV from normal and preeclamptic placentae is essential.

Acknowledgement

This study was supported by the Stichting Gezond Geboren and the German Research Foundation (Deutsche Forschungsgemeinschaft). C. Göhner and M. Weber have been partly financed by the Thuringian Ministry for Education, Science and Arts. C. Göhner was further financed by a fellowship of the Abel Tasman Talent Program of the University of Groningen.

References

[1] Y. Yuana, A. Sturk, R. Nieuwland, Extracellular vesicles in physiological and pathological conditions, Blood Rev. 27 (2013) 31–39. doi:10.1016/j.blre.2012.12.002.
[2] J. Southcombe, D. Tannetta, C. Redman, I. Sargent, The immunomodulatory role of syncytiotrophoblast microvesicles., PLoS One. 6 (2011) e20245. doi:10.1371/journal.pone.0020245.
[3] L. Mincheva-Nilsson, V. Baranov, Placenta-Derived Exosomes and Syncytiotrophoblast Microparticles and their Role in Human Reproduction: Immune Modulation for Pregnancy Success., Am. J. Reprod. Immunol. 72 (2014) 440–57. doi:10.1111/aji.12311.
[4] S.J. Gould, G. Raposo, As we wait: coping with an imperfect nomenclature for extracellular vesicles., J. Extracell. Vesicles. 2 (2013). doi:10.3402/jev.v2i0.20389.
[5] E. van der Pol, A.N. Böing, P. Harrison, A. Sturk, R. Nieuwland, Classification , Functions , and Clinical Relevance of Extracellular Vesicles, Pharmacol. Rev. 64 (2012) 676–705. doi:10.15407/biotech7.06.102.
[6] M. Szajnik, M. Czystowska, M.J. Szczepanski, M. Mandapathil, T.L. Whiteside, Tumor-derived microvesicles induce, expand and up-regulate biological activities of human regulatory T cells (Treg)., PLoS One. 5 (2010) e11469. doi:10.1371/journal.pone.0011469.
[7] M. Monguió-Tortajada, R. Lauzurica-Valdemoros, F.E. Borràs, Tolerance in organ transplantation: from conventional immunosuppression to extracellular vesicles., Front. Immunol. 5 (2014) 416. doi:10.3389/fimmu.2014.00416.
[8] G.P. Sacks, K. Studena, K. Sargent, C.W. Redman, Normal pregnancy and preeclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of sepsis., Am. J. Obstet. Gynecol. 179 (1998) 80–86. doi:S0002937898702546 [pii].
[9] T. Tilburgs, D. Schonkeren, M. Eikmans, N.M. Nagtzaam, G. Datema, G.M. Swings, et al., Human decidual tissue contains differentiated CD8+ effector-memory T cells with unique properties., J. Immunol. 185 (2010) 4470–7. doi:10.4049/jimmunol.0903597.
[10] T. Tilburgs, D.L. Roelen, B.J. van der Mast, J.J. van Schip, C. Kleijburg, G.M. de Groot-Swings, et al., Differential distribution of CD4(+)CD25(bright) and CD8(+)CD28(-) T-cells in decidua and maternal blood during human pregnancy., Placenta. 27 Suppl A (2006) S47–53. doi:10.1016/j.placenta.2005.11.008.
[11] M.T. Gervasi, T. Chaiworapongsa, N. Naccasha, P. Pacora, S. Berman, E. Maymon, et al., Maternal intravascular inflammation in preterm premature rupture of membranes., J. Matern. Fetal. Neonatal Med. 11 (2002) 171–175. doi:10.1097/00006254-200211000-00003.
[12] B.N. Melgert, F. Spaans, T. Borghuis, P.A. Klok, B. Groen, A. Bolt, et al., Pregnancy and preeclampsia affect monocyte subsets in humans and rats., PLoS One. 7 (2012) e45229. doi:10.1371/journal.pone.0045229.
[13] D.A. Somerset, Y. Zheng, M.D. Kilby, D.M. Sansom, M.T. Drayson, Normal human pregnancy is associated with an elevation in the immune suppressive CD25+ CD4+ regulatory T-cell subset., Immunology. 112 (2004) 38–43. doi:10.1111/j.1365-2567.2004.01869.x.
[14] V.R. Aluvihare, M. Kallikourdis, A.G. Betz, Regulatory T cells mediate maternal tolerance to the fetus., Nat. Immunol. 5 (2004) 266–71. doi:10.1038/ni1037.
[15] S. Saito, M. Sakai, Y. Sasaki, K. Tanebe, H. Tsuda, T. Michimata, Quantitative analysis of peripheral

blood Th0, Th1, Th2 and the Th1:Th2 cell ratio during normal human pregnancy and preeclampsia., Clin. Exp. Immunol. 117 (1999) 550–5.
[16] A.M. Borzychowski, B.A. Croy, W.L. Chan, C.W.G. Redman, I.L. Sargent, Changes in systemic type 1 and type 2 immunity in normal pregnancy and pre-eclampsia may be mediated by natural killer cells., Eur. J. Immunol. 35 (2005) 3054–63. doi:10.1002/eji.200425929.
[17] M. Kühnert, R. Strohmeier, M. Stegmüller, E. Halberstadt, Changes in lymphocyte subsets during normal pregnancy, Eur. J. Obstet. Gynecol. Reprod. Biol. 76 (1998) 147–151. doi:10.1016/S0301-2115(97)00180-2.
[18] A.L. Veenstra van Nieuwenhoven, A. Bouman, H. Moes, M.-J. Heineman, L.F.M.. de Leij, J. Santema, et al., Cytokine production in natural killer cells and lymphocytes in pregnant women compared with women in the follicular phase of the ovarian cycle, Fertil. Steril. 77 (2002) 1032–1037. doi:10.1016/S0015-0282(02)02976-X.
[19] M. Tong, L.W. Chamley, Placental Extracellular Vesicles and Feto-Maternal Communication, Cold Spring Harb. Perspect. Med. 5 (2015) 1–18. doi:10.1101/cshperspect.a023028.
[20] C. a R. Lok, J. a M. Van Der Post, I.L. Sargent, C.M. Hau, A. Sturk, K. Boer, et al., Changes in microparticle numbers and cellular origin during pregnancy and preeclampsia., Hypertens. Pregnancy. 27 (2008) 344–60. doi:10.1080/10641950801955733.
[21] C.W.G. Redman, D.S. Tannetta, R. a Dragovic, C. Gardiner, J.H. Southcombe, G.P. Collett, et al., Review: Does size matter? Placental debris and the pathophysiology of pre-eclampsia., Placenta. 33 Suppl (2012) S48–54. doi:10.1016/j.placenta.2011.12.006.
[22] J.A.M. van der Post, C.A.R. Lok, K. Boer, A. Sturk, I.L. IL Sargent, R. Nieuwland, et al., The functions of microparticles in pre-eclampsia., Semin. Thromb. Hemost. 37 (2011) 146–52. doi:10.1055/s-0030-1270342.
[23] T. Chaiworapongsa, P. Chaemsaithong, L. Yeo, R. Romero, Pre-eclampsia part 1: current understanding of its pathophysiology., Nat. Rev. Nephrol. 10 (2014) 466–80. doi:10.1038/nrneph.2014.102.
[24] J.R. Prins, H.M. Boelens, J. Heimweg, S. Van der Heide, A.E. Dubois, A.J. Van Oosterhout, et al., Preeclampsia is associated with lower percentages of regulatory T cells in maternal blood., Hypertens. Pregnancy. 28 (2009) 300–11. doi:10.1080/10641950802601237.
[25] J. Mjosberg, G. Berg, M.C. Jenmalm, J. Ernerudh, FOXP3+ Regulatory T Cells and T Helper 1, T Helper 2, and T Helper 17 Cells in Human Early Pregnancy Decidua, Biol. Reprod. 82 (2010) 698–705. doi:10.1095/biolreprod.109.081208.
[26] D. Schonkeren, M.-L. van der Hoorn, P. Khedoe, G. Swings, E. van Beelen, F. Claas, et al., Differential distribution and phenotype of decidual macrophages in preeclamptic versus control pregnancies., Am. J. Pathol. 178 (2011) 709–17. doi:10.1016/j.ajpath.2010.10.011.
[27] D. Goswami, D.S. Tannetta, L. a Magee, a Fuchisawa, C.W.G. Redman, I.L. Sargent, et al., Excess syncytiotrophoblast microparticle shedding is a feature of early-onset pre-eclampsia, but not normotensive intrauterine growth restriction., Placenta. 27 (2006) 56–61. doi:10.1016/j.placenta.2004.11.007.
[28] D. Tannetta, M. Mackeen, B. Kessler, I. Sargent, C. Redman, OS045. Multi-dimensional protein identification technology analysis of syncytiotrophoblast vesicles released from perfused preeclampsia placentas., Pregnancy Hypertens. 2 (2012) 201–2. doi:10.1016/j.preghy.2012.04.046.
[29] J.E. Gumperz, S. Miyake, T. Yamamura, M.B. Brenner, Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining., J. Exp. Med. 195 (2002) 625–36.
[30] B. Fu, Z. Tian, H. Wei, Subsets of human natural killer cells and their regulatory effects, Immunology. 141 (2014) 483–489. doi:10.1111/imm.12224.
[31] C. Göhner, M. Weber, D.S. Tannetta, T. Groten, T. Plösch, M.M. Faas, et al., A New Enzyme-linked Sorbent Assay (ELSA) to Quantify Syncytiotrophoblast Extracellular Vesicles in Biological Fluids, Am. J. Reprod. Immunol. 73 (2015) 582–588. doi:10.1111/aji.12367.
[32] T. Tilburgs, D.L. Roelen, B.J. van der Mast, G.M. de Groot-Swings, C. Kleijburg, S.A. Scherjon, et al., Evidence for a Selective Migration of Fetus-Specific CD4+CD25bright Regulatory T Cells from the Peripheral Blood to the Decidua in Human Pregnancy, J. Immunol. 180 (2008) 5737–5745. doi:10.4049/jimmunol.180.8.5737.

[33] A. van Egmond, C. van der Keur, G.M.J.S. Swings, S.A. Scherjon, F.H.J. Claas, The possible role of virus-specific CD8(+) memory T cells in decidual tissue., J. Reprod. Immunol. 113 (2015) 1–8. doi:10.1016/j.jri.2015.09.073.
[34] M.G. Ruocco, G. Chaouat, L. Florez, A. Bensussan, D. Klatzmann, Regulatory T-cells in pregnancy: historical perspective, state of the art, and burning questions., Front. Immunol. 5 (2014) 389. doi:10.3389/fimmu.2014.00389.
[35] J.H. Rowe, J.M. Ertelt, L. Xin, S.S. Way, Pregnancy imprints regulatory memory that sustains anergy to fetal antigen., Nature. 490 (2012) 102–6. doi:10.1038/ nature11462.
[36] X. Cao, S.F. Cai, T.A. Fehniger, J. Song, L.I. Collins, D.R. Piwnica-Worms, et al., Granzyme B and perforin are important for regulatory T cell-mediated suppression of tumor clearance., Immunity. 27 (2007) 635–46. doi:10.1016/j.immuni.2007.08.014.
[37] D. Chowdhury, J. Lieberman, Death by a thousand cuts: granzyme pathways of programmed cell death., Annu. Rev. Immunol. 26 (2008) 389–420. doi:10.1146/annurev.immunol.26.021607.090404.
[38] E. a P. Steegers, P. von Dadelszen, J.J. Duvekot, R. Pijnenborg, Pre-eclampsia., Lancet. 376 (2010) 631–44. doi:10.1016/S0140-6736(10)60279-6.
[39] B. Sibai, G. Dekker, M. Kupferminc, A.S. Way, Pre-eclampsia., Lancet. 365 (2005) 785–99. doi:10.1016/S0140-6736(05)17987-2.